# THE BINDING OF *ESCHERICHIA COLI* ENDOTOXIN TO ISOLATED RAT HEPATOCYTES

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## 1. Introduction

The mechanisms through which bacterial endotoxins exert their biological activity is not well understood. A characteristic property of lipopolysaccharides (LPS) is their marked heterogeneity in terms of physical state, molecular size and charge. Also, LPS contain varying amounts of impurities according to the different methods of extraction and this might explain the various results reported with different preparations.

Investigations in the field of bacterial endotoxins suggested the major responsibility of the lipid component for the endotoxic activities of LPS [1–4]. Some types of interactions between LPS and cell membranes have been reported for red cells [5], lymphocytes [6], granulocytes [7] and platelets [8].

The cell membrane of eukaryotic cells contains significant amounts of phospholipids that probably play a role in the binding of LPS to cell membranes [9]; thus, the variation in the phospholipid composition among cell types might account for the specificity in the biological reactions elicited by LPS [10–12] and it has been reported that there is a direct relation between the susceptibility of different strains of mice to the lethal effects of *Salmonella* endotoxin and the affinity of their red cells for endotoxin [13].

However, it is generally accepted that liver exerts a LPS clearance function with the participation of both Kupffer cells [14–17] and parenchymal cells [18]. Also, there is now general agreement that, in the liver, endotoxemia causes hepatocyte mitochondrial alterations that can be characterized by both morphologic and functional alterations [19,20].

This paper is concerned with the binding properties of $^{14}$C-labelled lipopolysaccharide ($^{14}$C-LPS) to isolated hepatocytes from rats.

## 2. Experimental

Endotoxin labelled with $^{14}$C was obtained from *Escherichia coli* 0111a,0111b: K58:H21 (ATCC) grown in a medium with [$^{14}$C]glucose as the only carbon source. The extraction of $^{14}$C-LPS was carried out according to Romanowska's modification [21] of the general method of Westphal. Specific activity was 2.23 $\mu$Ci/mg; $A_{260}/A_{240}$ was determined for purity verification.

Hepatocytes were isolated from male rats (Sprague-Dawley, 250–300 g, fasted over 20 h with water *ad libitum*), by the perfusion technique in [22] using collagenase (Sigma, St Louis MO; type IV, 170 U/mg) (30 mg) in 131 ml Krebs–Ringer–bicarbonate solution, without $Mg^{2+}$. The cell suspension was purified by repeated centrifugation at 165 g for 20 s.

Cell viability was monitored by trypan blue exclusion and cell preparations had 85–90% viability. Gluconeogenesis from lactate was measured in isolated cells incubated with 10 mM substrate in Krebs–Ringer–bicarbonate solution ($10^6$ cells/500 $\mu$l). Glucose levels were determined in the supernatants enzymically using glucoseoxidase and peroxidase (Kit Carlo Erba).

Control of structure was carried out by electron microscopy of thin sections of Epon-embedded cell suspensions, cut on an LKB Ultrotome III ultramicrotome, and examined in a Jeol 100 electron microscope.

Stimulation of gluconeogenesis by glucagon (Novo, Spain) was determined by incubating the hormone with hepatocytes in various experimental conditions.

The standard binding assay was based on the method in [23] and consisted of incubating hepatocytes at 25°C in complete Krebs–Ringer–bicarbonate solution containing $^{14}$C-LPS. Hepatocyte–LPS

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incubates were shaken to ensure adequate interaction between the cells and the medium. Incubation volume 500 μl; cells 2–5 × $10^5$; and $^{14}$C-LPS 1–100 μg. Aliquots of the suspensions were removed from the incubation flask and filtered through Whatman GF/C filters at different times according to the experiment. Data generated employing labelled endotoxin alone have been designated as total binding [24]. Such uptake contained receptor and non-receptor cellular binding. The non-specifically bound LPS was determined by either the method in [25] where a high concentration of unlabelled LPS was previously added, or by the displacement method [26] where the component of radioactive ligand not displaced by high concentrations of LPS was considered to be non-specifically bound. Subtraction of non-specifically bound LPS from the total radioactive uptake generated specific binding curves.

Rebinding experiments using $^{14}$C-LPS from supernatants of previous binding studies showed the stability of LPS at the incubation conditions. The stability of receptors was demonstrated by incubating a high concentration of unlabelled LPS either previously to, or simultaneously with, the addition of $^{14}$C-LPS to the hepatocytes; the radioactive incorporation was very similar in both procedures.

Unlabelled endotoxin was from *E. coli* 0111:B4 obtained as in [27] and supplied by Difco.

Incorporation of radioactivity was measured in a Packard Tri-Carb 3255 scintillation counter using 15 ml of the mixture: naphtalene, 150 g; PPO, 10.5 g; POPOP, 0.4 g; dioxane to 1.5 l.

## 3. Results and discussion

The standard binding assay associated hepatocytes with $^{14}$C-LPS at 25°C for different time intervals (3–75 min), after which unbound lipopolysaccharide was removed by filtration. The association of $^{14}$C-LPS with hepatocytes was initially rapid and an apparent maximum binding was observed from ~30 min (fig.1). This incorporation was shown proportional to both the $^{14}$C-LPS concentration and the number of cells used for the experiment.

The binding properties of lipopolysaccharide after being exposed for 15 min to the cells were assayed using the supernatant in a new hepatocyte incubation. Table 1 shows the isotope incorporation data in both incubation experiments; these data are in agreement with the dose of lipopolysaccharide used in each experiment and confirm its stability under the incubation conditions.

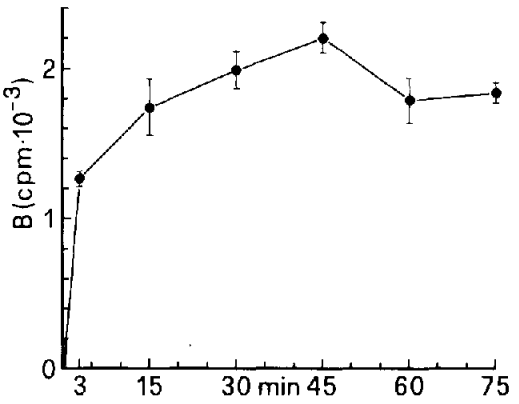


Fig.1. Time course of association of $^{14}$C-LPS with hepatocytes. Hepatocytes (5 × $10^5$ cells) were incubated with 2.8 μg $^{14}$C-LPS/500 μl (spec. act. 2.23 μCi/mg) in a series of flasks that were shaken for appropriate times. Each point represents the mean of triplicate determinations of the total binding.

The presence of 1% bovine serum albumin in the hepatocyte incubation medium influenced negatively the uptake of $^{14}$C-LPS after 25 min (table 2). Thus,

Table 1
Rebinding experiment using the supernatant of a $^{14}$C-LPS preincubation with 2 × $10^5$ hepatocytes/500 μl

| Conditions | $^{14}$C-LPS doses | | Incorp. (μg) |
|---|---|---|---|
| | (μg) | (ng/μl) | |
| Incubation 1 (2 × $10^5$ cells/500 μl) | 2.47 | 4.9 | 0.80 |
| Incubation 2 (2 × $10^5$ cells/200 μl of supernatant incubation 1 | 0.36 | 0.9 | 0.16 |

Table 2
Influence of 1% bovine serum albumin on the binding of $^{14}$C-LPS, at different concentrations, on hepatocytes

| Incubation conditions | Time (min) | $^{14}$C-LPS (μg) | Incorp. (μg) |
|---|---|---|---|
| Control | 25 | 20 | 1.140 ± 0.016 |
| | 25 | 80 | 2.860 ± 0.015 |
| +1% albumin | 25 | 20 | 0.340 ± 0.002 |
| | 25 | 80 | 1.080 ± 0.015 |

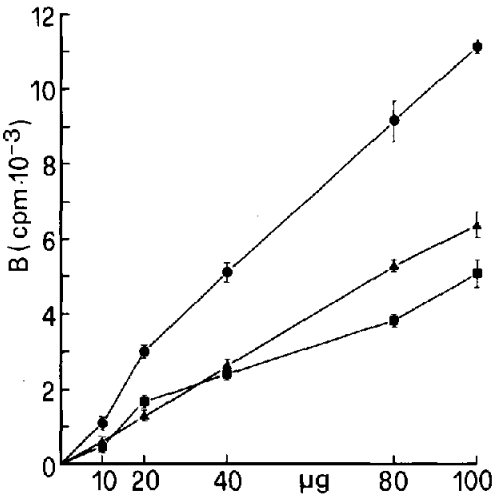


Fig.2. Association of $^{14}$C-LPS with hepatocytes. The total binding curve (—•—) was determined using 2 × $10^5$ cells and 10–100 μg $^{14}$C-LPS (spec. act. 0.225 μCi/mg). The non-specific points (—▪—) were determined by preincubation with 400 μg LPS of 2 × $10^5$ cells, for 25 min at 25°C. The specific binding curve (—▲—) was calculated by difference.

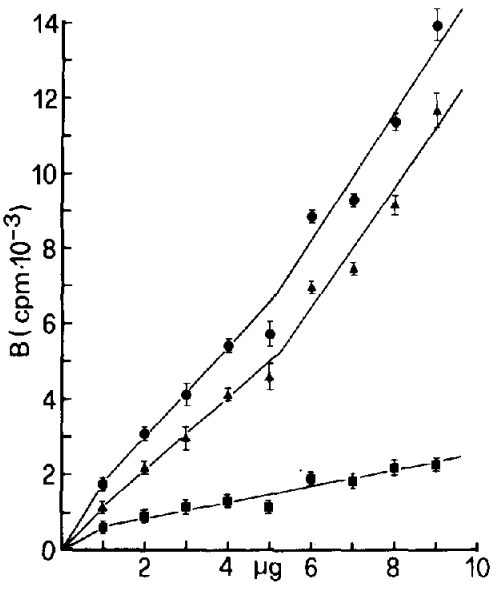


Fig.3. Association of $^{14}$C-LPS with hepatocytes. The non-specific binding was determined by preincubation of 2 × $10^5$ cells with 2 mg LPS, for 5 min, at 25°C. Symbols for total, specific and non-specific binding as in fig.2.

all experiments were carried out in the absence of albumin.

The saturation assays of hepatocyte suspensions were accomplished over 10–100 μg $^{14}$C-LPS/2 × $10^5$ cells. Preincubation with unlabelled LPS for 25 min decreased the subsequent uptake of $^{14}$C-LPS by hepatocytes. The binding capacity decreased as the LPS concentration to which cells were pre-exposed increased (fig.2). In total binding systems, the concentration of $^{14}$C-LPS accumulated followed a linear saturation up to 100 μg $^{14}$C-LPS. The unspecific binding results were independent of the relative time of addition of both labelled and unlabelled LPS; thus, when the addition to cells of both, labelled and unlabelled LPS, was carried out simultaneously, the $^{14}$C-LPS binding was similar to that when cells were preincubated with the unlabelled LPS previously to the addition of the labelled one.

With the aim of optimizing the specific binding conditions, similar experiments were carried out with hepatocytes exposed to lower concentrations of $^{14}$C-LPS at shorter incubation times (5 min). In fact, results given in fig.3 show that these incubation conditions minimize the non-specific binding and that it is linear with the $^{14}$C-LPS concentration. However, the specific binding curve of $^{14}$C-LPS to hepatocytes is not consistent with the existence of specific sites on cells.

The receptor–LPS affinity was also assayed by the displacement method incubating hepatocytes, at 25°C for 15 min, with different amounts of $^{14}$C-LPS; the cells were afterwards incubated, for 25 min, with an excess of native LPS (*E. coli* 0111:B4). A saturation experiment was accomplished simultaneously at the same cell no./$^{14}$C-LPS ratio. Table 3 shows that the amounts of radioactive ligand bound to hepatocytes, and not displaced by high concentrations of native LPS, were quite similar to those bound to the cells

Table 3
Saturation and displacement binding assays with $^{14}$C-LPS and 2 × $10^5$ hepatocytes/500 μl, at 25°C for 15 min

| Assay | $^{14}$C-LPS (μg) | $^{14}$C-LPS incorp. (μg) |
|---|---|---|
| Saturation | 20 | 6.47 ± 0.52 |
| | 40 | 10.80 ± 0.37 |
| Displacement | 20 | 6.15 ± 0.10 |
| | 40 | 10.97 ± 0.45 |

Displacement was done with 400 μg *E. coli* 0111:B4 LPS for 25 min

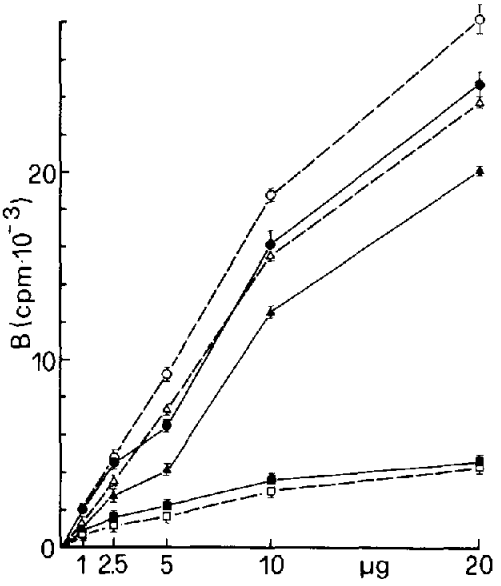


Fig.4. Influence of hepatocyte viability on the total (●,○), specific (▲,△) and non-specific (■,□) binding of $^{14}$C-LPS. Viability was 76% (——) and 7.3% (– – –). The non-specific binding as in fig.3.

under saturating conditions. Thus, high concentrations of LPS did not decrease the amount of $^{14}$C-LPS retained by hepatocytes by competing for the affinity sites. This suggested that an unspecific high affinity process is responsible for the binding of lipopolysaccharide to hepatocytes.

Figure 4 shows the capacity of hepatocytes to accumulate $^{14}$C-LPS as a function of cell viability, as shown by trypan blue exclusion. The viability of a hepatocyte suspension was physically damaged by slow treatment in a Dounce homogenizer and these cells were also assayed for the retention of the lipopolysaccharide. In fact, the cellular binding capacity of $^{14}$C-LPS by both types of cells, of 75% and 7% viability, resulted in identical retentions, although the low viability cells show total and specific binding levels higher than those of normal cells. Each of these observations is consistent with the interpretation of an absence of specific receptor sites in the membrane; the higher level of incorporation by the damaged cells lends assurance that a higher occupancy by the labelled ligand is due to some extent to membrane fragmentation, as has been shown by electron microscopy.

The decrease of hepatocyte viability as a consequence of the cellular damage has been related to the

Table 4
Relationship between morphological viability of hepatocytes and gluconeogenesis from 10 mM lactate stimulated by $10^{-6}$ M glucagon

| Viability | Time (min) | nmol glucose/ $10^6$ cells |
|---|---|---|
| 86% | 30 | 53.3 ± 3.8 |
| | 60 | 195.5 ± 6.2 |
| | 90 | 340.5 ± 3.8 |
| 42% | 30 | 11.9 ± 1.0 |
| | 60 | 42.3 ± 1.0 |
| | 90 | 74.7 ± 3.8 |

biosynthesis of glucose from lactate induced by glucagon. Table 4 indicates the correlation between both morphological and biochemical assays and, therefore, the LPS-binding effect is independent of the cell viability.

All these results suggest the non-existence in hepatocytes of functional specific receptors for the lipopolysaccharide in the cell membrane although the lipopolysaccharide binds to particular ingredients of the membrane bilayer and induces a destabilization of the membrane. This hypothesis agrees with [28–30] and with data obtained using a lipoprotein as the target structure of the *E. coli* lipopolysaccharide [31].

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